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Award Number: W81XWH-05-1-0446

TITLE: Therapeutic Conversion of Viability Promoting MCL1 to Death-Inducing Forms: A Novel Strategy for Breast Cancer

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REPORT DATE: July 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-07-2007	Final	15 Jun 2005 – 14 Jun 2007

4. TITLE AND SUBTITLE

Therapeutic Conversion of Viability Promoting MCL1 to Death-Inducing Forms: A Novel Strategy for Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0446

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Dartmouth College
Hanover, NH 03755-1404

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES –Original contains colored plates: ALL DTIC reproductions will be in black and white.

14. ABSTRACT

The BCL2 family member MCL1 is expressed in breast cancer cells in its full-length, anti-apoptotic form. The goal of this project was to induce conversion of MCL1 to pro-apoptotic forms as a means of enhancing the death of these cells. The approach was identify means of inducing alternate splicing of MCL1 using antisense oligonucleotides, since splice variants are known to promote cell death rather than cell survival. We identified reagents and conditions that result in decreased expression of the antiapoptotic MCL1L protein and increased expression of proapoptotic splice variants. These splice-switching agents inhibited tumor cell growth, and did so in a more sustained fashion than siRNA directed against MCL1L. Reagents that induce splice switching of MCL1 thus have promise for further development for the treatment of breast cancer.

15. SUBJECT TERMS
No subject terms provided.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	12	**19b. TELEPHONE NUMBER** *(include area code)*

INTRODUCTION

The BCL2 family member MCL1 is expressed in breast cancer cells in its anti-apoptotic form, $MCL1_L$. The goal of this project was to induce conversion of MCL1 to the much shorter pro-apoptotic forms as a means of enhancing the death of these cells. The approach is to induce alternate splicing of MCL1, since splice variants are known to promote cell death rather than cell survival (1). MCL1 is encoded in 3 exons, which are normally spliced together to yield the full-length gene product[1]. This full-length form of MCL1 contains the BCL2 Homology domains BH1, BH2, and BH3, which are important for the promotion of viability. Alternate splice variants of MCL1 cause cell death. For example, in $MCL1_{s/\Delta TM}$, exon 1 is spliced to exon 3 with skipping of exon 2. This truncated variant contains BH3 (in exon 1) but not other BH domains. $MCL1_{s/\Delta TM}$ thus behaves like the death-inducing BH3-only members of this family. In this fashion, differential splicing can convert MCL1 from anti- to pro-apoptotic forms. The purpose of this grant was to devise approaches for targeting the conversion of the full-length anti-apoptotic form of MCL1 to pro-apoptotic forms, to assess this as a potential therapeutic strategy. Our approach was to use antisense oligonucleotides to attempt to induce alternate splicing of MCL1. We also monitored the effects of manipulating the expression of these MCL1 isoforms on cell viability. The induction of MCL1 splice-switching may represent a novel, feasible, and efficacious approach for targeted gene therapy for breast cancer.

RESEARCH ACCOMPLISHED

I. Identify oligonucleotides and conditions that optimize alternative splicing, assess alternative splicing by PCR and Western blotting, and assess effects on cell growth and viability.

A. Conditions for the use of splice-site directed antisense oligonucleotides, and the effects of these agents on MCL1 mRNA and protein expression, have been determined.

We designed antisense phosphorothioate 2'-O-methyl oligonucleotides directed against the upstream and downstream splice sites flanking MCL1 exon 2, as described in the previous progress report. These reagents were tested in the NT2 cell line, where essentially the entire cell population can be transiently transfected using oligofectamine. Application of these antisense oligonucleotides at a concentration of 100 nM each resulted in an increase in the expression of $MCL1_{s/\Delta TM}$,but not $MCL1_L$, mRNA (Fig. 1, upper two photographs). Optimization of the PCR

conditions for detection of $MCL1_{s/\Delta TM}$ indicated that the extent of the increase was at least 6-fold (Fig. 1, third photograph). Antisense oligonucleotides directed against sequence within intron 1 or intron 2, used as controls, did not produce a dramatic effect. This effect of splice site-directed antisense oligonucleotides was concentration-dependent, with extensive conversion to $MCL1_{s/\Delta TM}$ occurring at concentrations of 100-200 nM (Fig. 2A). An increase in the expression of proapoptotic forms of the MCL1 protein was accompanied by a decrease in the expression of the antiapoptotic $MCL1_L$ protein (Fig. 2B), despite the fact that the mRNA for the latter was still in evidence. In sum, these reagents are capable of inducing a dual effect -- increasing proapoptotic, and decreasing antiapoptotic, forms of MCL1.

B. MCL1 splice-switching reagents have been shown to produce sustained inhibition of tumor cell growth.

Along with the above effects on expression of the various forms of MCL1, the splice site-direct antisense oligonucleotides inhibited tumor cell growth and viability (Fig. 3). This effect was sustained over the 3 day assay period with the 100 mM concentration (Fig. 4); here, although the tumor cell population was not completely killed, cell number did not increase. In this respect, the splice-site directed reagents provided an advantage over more standard short interfering RNA (siRNA) treatment. siRNAs reduce the expression of viability-promoting $MCL1_L$ (Fig. 5A) but are not designed to cause conversion to death-inducing forms. Furthermore, inhibition of expression of $MCL1_L$ was transient (Fig. 5B), and inhibition of tumor cell growth was likewise transient such that regrowth was apparent from day 1 to day 3 (Fig. 6) even at the highest siRNA concentration tested. Thus, the results with splice switching reagents are very encouraging as these were more effective than siRNAs in this *in vitro* system.

C. Expression of different forms of MCL1 has been monitored in breast cancer cells.

In addition to the above studies aimed at inducing splice-switching with antisense oligonucleotides, we further examined expression of the various forms of MCL1 in breast cancer cells. This was done in the presence or absence of a variety of agents active in these cells. We previously observed that many breast cancer lines express only low or barely detectable levels of the proapoptotic forms of the MCL1 protein, $MCL1_{s/\Delta TM}$ and $MCL1_{exon1}$. Further investigation has revealed that detectable expression is present in some lines, as seen in MCF-7 (Fig. 7A). Interestingly, exposure of these cells to beta-estradiol plus EGF resulted in decreased expression of $MCL1_L$ and increased expression of $MCL1_{s/\Delta TM}$. The proapoptotic forms were not detectable or only very faintly detectable in Western blots with MD-MBA231 and MD-MBA468 (Fig. 7A), although very low levels of the $MCL1_{s/\Delta TM}$ mRNA were present in these lines (not shown). Proapoptotic forms were readily detectable in MD-MBA436 (provided by Dr. K. Keyomarsi), which also expressed $MCL1_{ATG1}$ (a product representing initiation at MCL1 Met 111). Proapoptotic forms were not detected in SKBR3 cells, but exposure of these cells to TPA resulted in increased expression of $MCL1_L$ and $MCL1_{s/\Delta TM}$ (Fig. 7A). In MCF-7 cells, the chemotherapeutic agents paclitaxel and etoposide cause increased expression of $MCL1_{exon1}$ along with decreased expression of $MCL1_L$ (Fig. 7B). Overall, exogenous agents can affect the expression of the different forms of MCL1 in breast cancer cells, and some agents active in this disease may act, in part, through inducing changes in the expression of the various splice variants of MCL1.

II. Apply nucleic acid reagents as developed above *in vivo*.

Because splice switching in vitro was not achieved easily, we focused our efforts on optimizing this in cells in culture as it would not make sense to apply a non-optimized approach *in vivo*.

KEY RESEARCH ACCOMPLISHMENTS

1. Splice switching antisense oligonucleotides, when applied at concentrations of 100-200 nM, were found to result in decreased expression of the antiapoptotic $MCL1_L$ protein, and increased expression of proapoptotic forms of MCL1.

2. The splice switching reagents were found to cause sustained inhibition of cell growth. In this respect, they demonstrated a more pronounced effect than MCL1 siRNAs, which caused only transient inhibition of expression of $MCL1_L$ with no effect on proapoptotic forms, and resulted in only transient inhibition of cell growth.

3. Some currently available agents were found cause decreased expression of antiapoptotic $MCL1_L$ and increased expression of pro-apoptotic forms in breast cancer cells, suggesting that this is one action of these agents. This provides a further rationale for continued investigation of the effects of the splice switching reagents by themselves, to determine whether they have an improved profile of efficacy, potency, and potential for side effects as compared to conventional agents.

FUNDING APPLIED FOR

BC050579: Targeted Therapy of Breast Cancer by Splice-switching of Anti-apoptotic MCL1 to Death-inducing Form

BC053432: Ultrasound-activated Micro/Nanobubbles for the Delivery of siRNAs that Target Oncogenes and Survival-promoting Genes Expressed in Breast Tumors

Personnel Receiving Salary From This Grant:
Ruth W. Craig, Principle Investigator
Samuel L. Casella, Research Technician

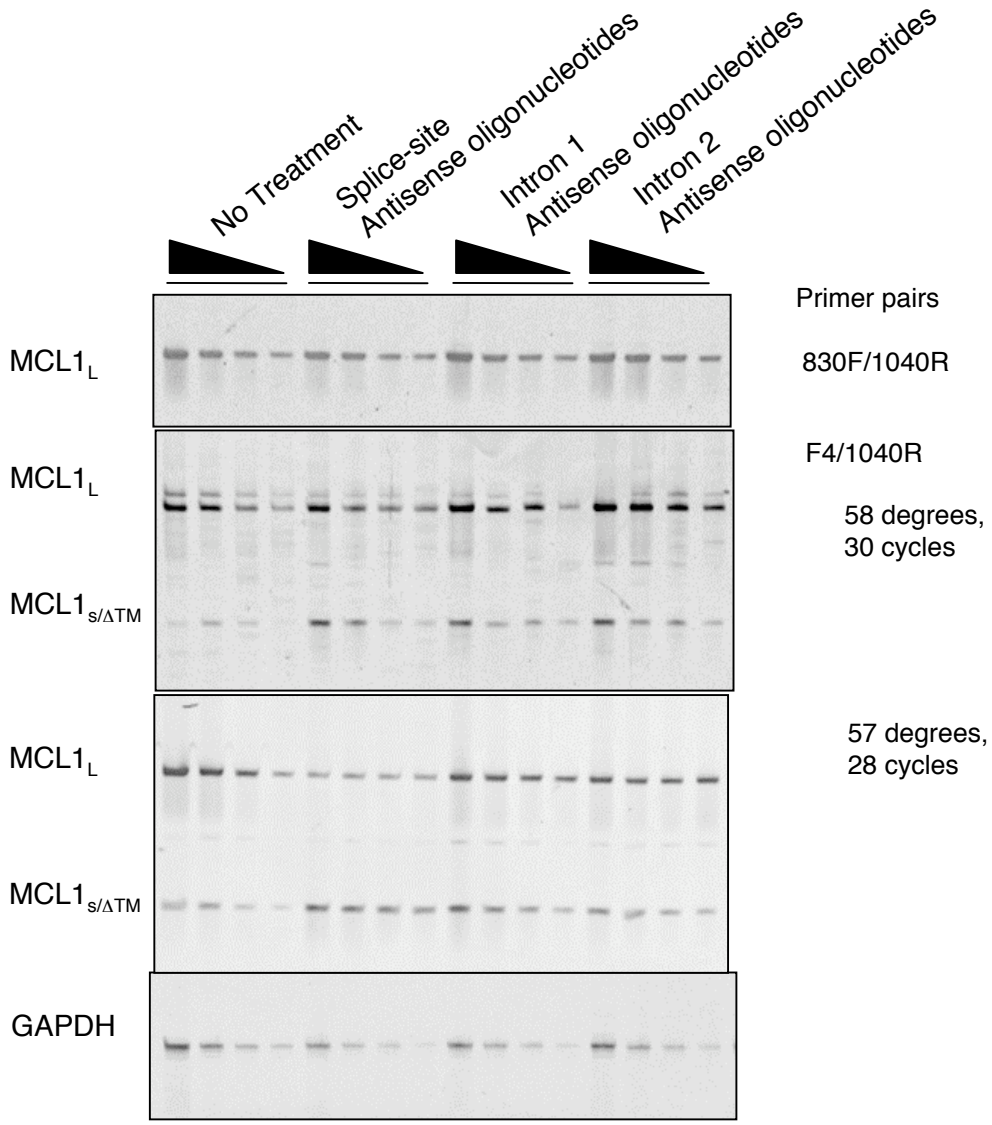
CONCLUSIONS

The results of these studies are extremely encouraging in that the splice-switching reagents were found to induce both downregulation of antiapoptotic $MCL1_L$, and increased expression of short pro-apoptotic MCL1 variants. Probably because of these dual effects, the introduction of splice-switching reagents (by lipofection) resulted in inhibition of tumor cell growth that was more sustained than that obtained with MCL1 siRNAs. The approach of inducing splice switching therefore shows promise for application to breast cancers exhibiting abundant expression of $MCL1_L$.

REFERENCES

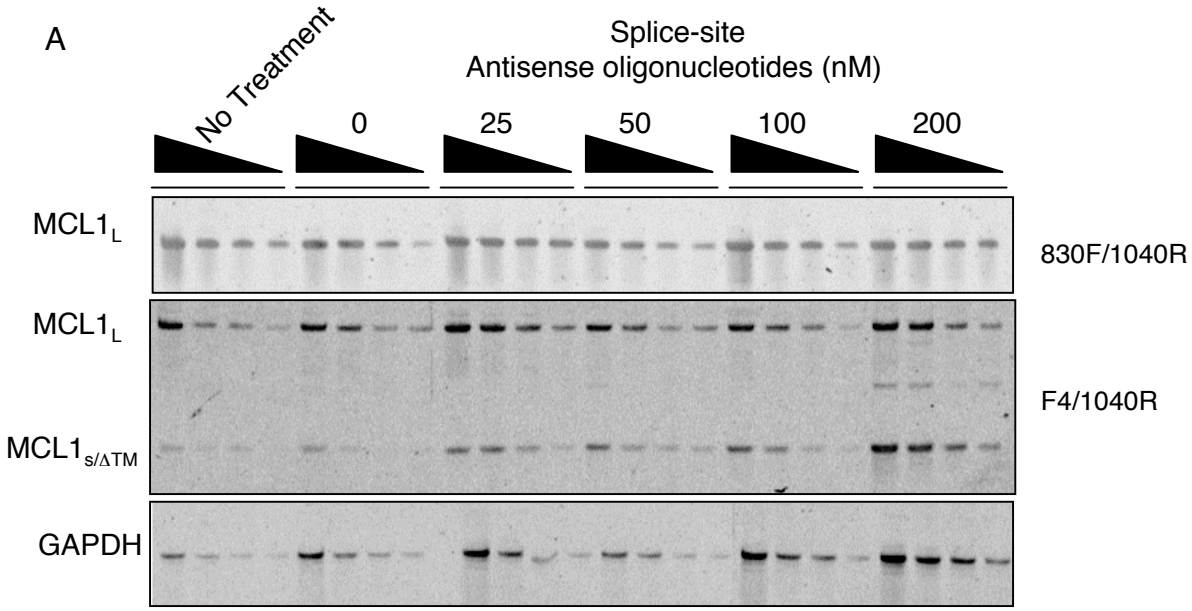
1. Bingle CD, Craig RW, Hanks BM et al. Exon skipping in MCL1 results in a BCL2 homology domain 3 (BH3)-only gene product that promotes cell death. J Biol Chem 2000; 275: 22136-22146.

Fig. 1. Increase in Expression of $MCL1_{s/\Delta TM}$ Induced by Splice Site-directed MCL1 Oligonucleotides



$MCL1_L$/GAPDH:	1	0.9	1.6	1.1
$MCL1_{s/\Delta TM}$/GAPDH:	1	6.4	2.2	1.2
$MCL1_{s/\Delta TM}$/MCL_L:	1	7.2	1.3	1.2
Dye-excluding cells (day 1):	88%	41%	59%	58%

Fig. 2. Splice-site Directed Antisense Oligonucleotides Increase Expression of Proapoptotic MCL1 Splice Variants and Decrease Expression of the Antiapoptotic $MCL1_L$ Protein



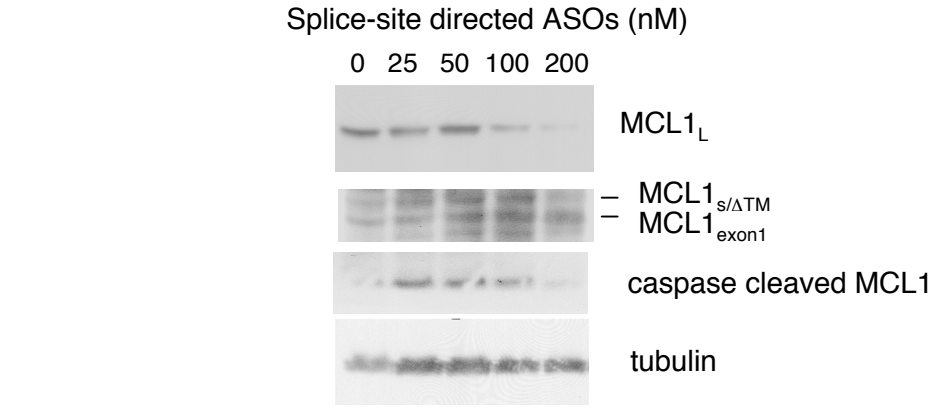


Fig. 3. Splice-site Directed Oligonucleotides Promote Cell Death

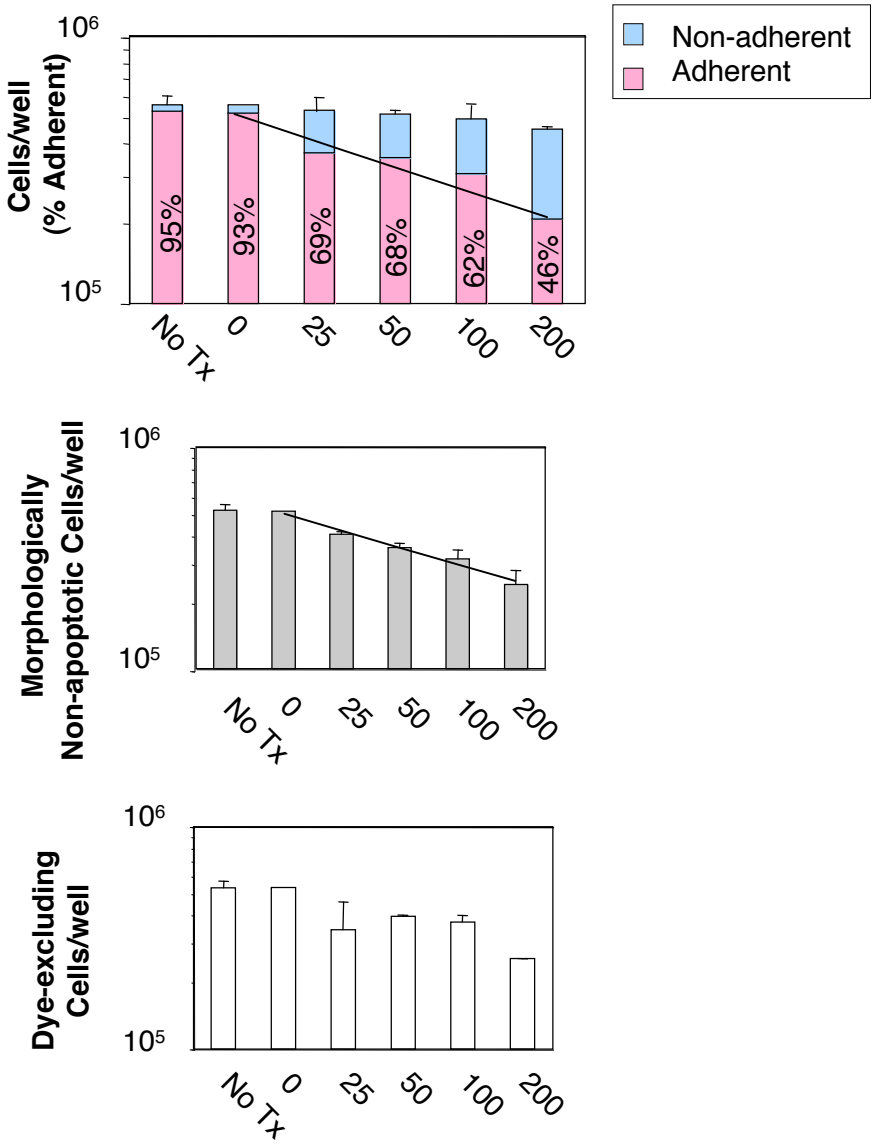
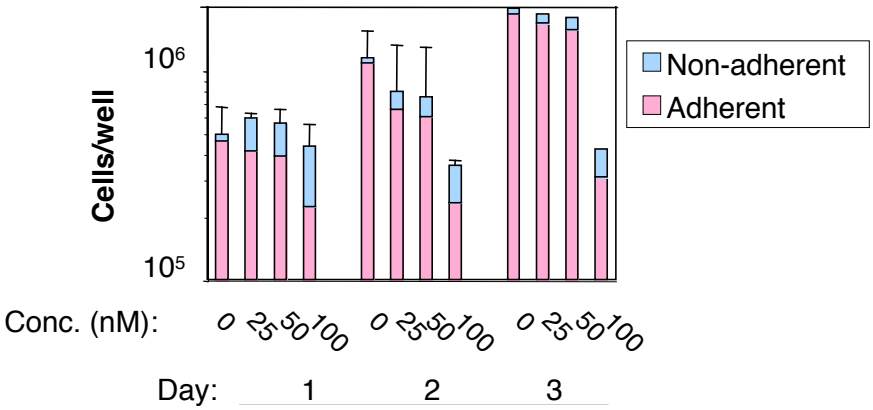
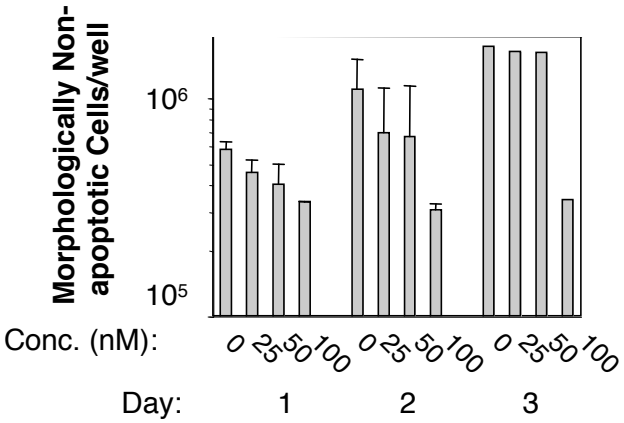


Fig. 4. Splice-site Directed MCL1 Oligonucleotides Produce Sustained Inhibition of Tumor Cell Growth





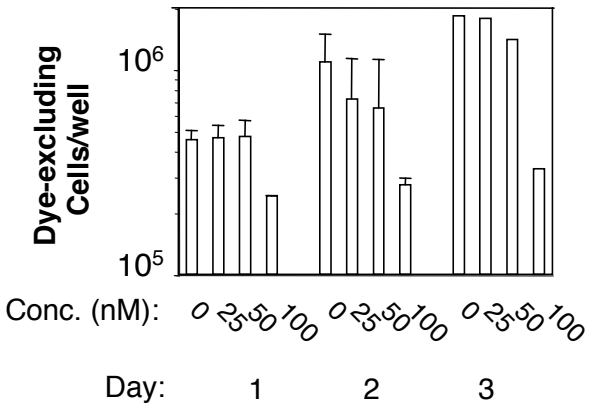
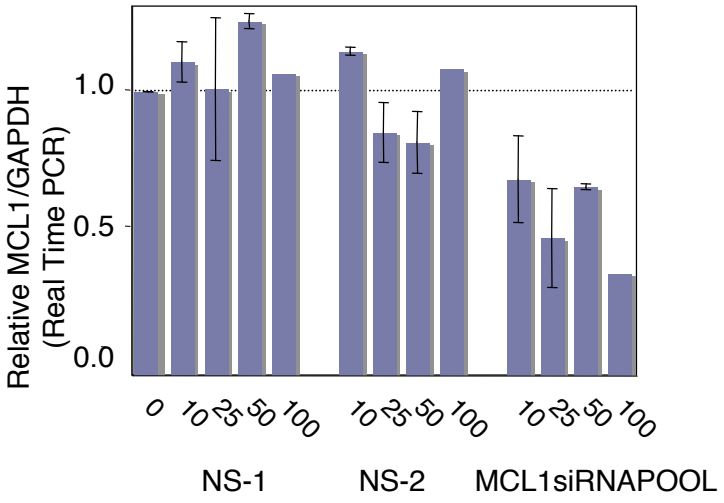


Fig. 5. MCL1 siRNA Produces only Transient Suppression of MCL1 Expression

A



B

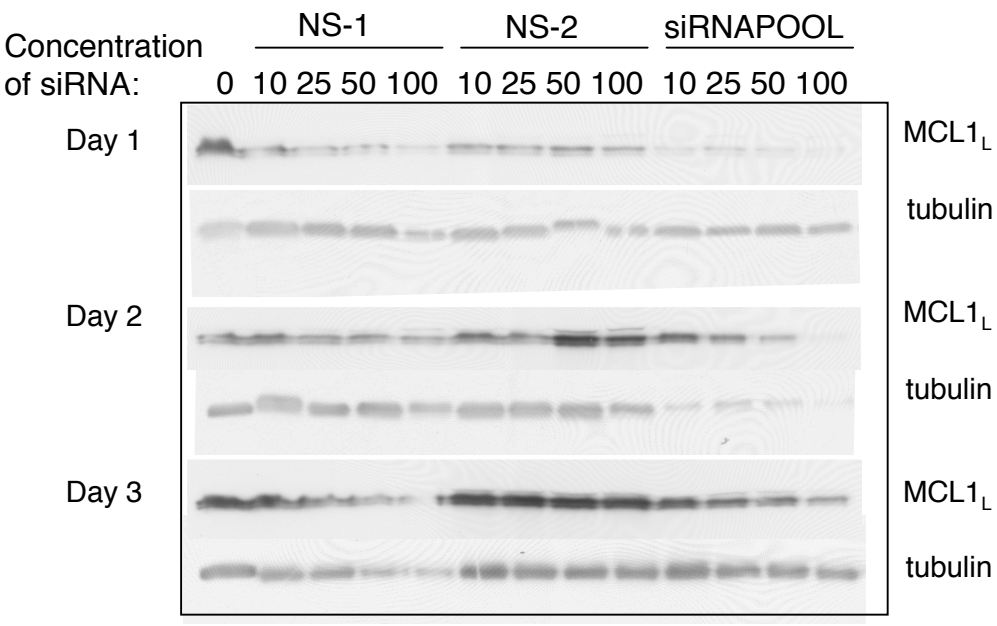


Fig. 6. MCL1 siRNA allows Tumor Cell Outgrowth

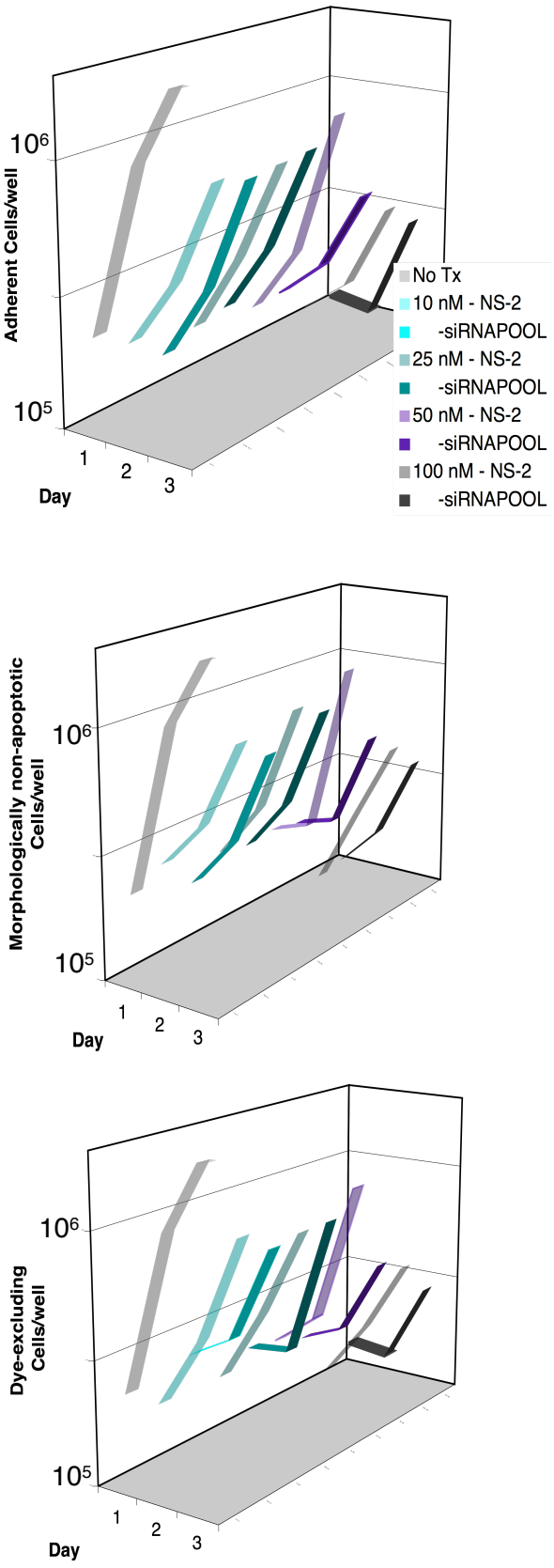


Fig. 7. Agents Used in Breast Cancer Decrease Expression of $MCL1_L$ and Increase Expression of Proapoptotic Forms